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Chromatographic purification of human α_1 proteinase inhibitor from dissolved Cohn fraction IV-1 paste

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Abstract

A novel chromatographic process for purification of α_1 proteinase inhibitor (α_1-PI) from Cohn fraction IV-1 paste is described. This process has been successfully scaled up to 50-l columns. It involves DEAE chromatography, sulfopropyl (S) cation chromatography, tri-*n*-butyl phosphate (TNBP)–cholate treatment, a second S cation chromatography, freeze–drying and dry-heat. The process has been optimized for purity, yield, lipid removal, chemical usage and water consumption. Filtration after TNBP–cholate treatment plays a key role in ensuring a low lipid content in the final product. Pre-equilibration with high salt buffer is necessary to reduce the water consumption significantly during the ion-exchange chromatography equilibration step. The final product is approximately 95% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, with a 64% to 70% yield from IV-1 paste. © 1998 Elsevier Science B.V.

Keywords: Cohn fraction IV-1 paste; Preparative chromatography; Proteinase inhibitor; Enzyme inhibitors; Proteins

1. Introduction

α_1 Proteinase inhibitor (α_1-PI), a serine proteinase inhibitor that forms stable stoichiometric complexes with serine proteinases (e.g., trypsin, chymotrypsin and elastase) [1], is useful in treating emphysema, a lung disease caused by an α_1-PI deficiency [2–4]. α_1-PI exhibits a molecular mass of approximately 52 000 [3], has 394 amino acids [3], a single cysteine residue [5], and three carbohydrate attachment sites [6]. The reactive loop of uncleaved α_1-PI has been demonstrated by X-ray crystallography [7] to have a distorted helical conformation, but no pre-insertion into the β-sheet. The crystal structure of a post-complex form of α_1-PI was examined by Lobermann et al. [8].

Published methods for the purification of α_1-PI from human plasma essentially use either chromatography or a combination of Cohn fractionation and chromatography methods [9–23].

For example, Schultze and Marbach [9] prepared α_1-PI from human plasma using carboxymethyl ion-exchange chromatography at pH 5.0 to 5.5 and a buffer salt concentration of 0.005 to 0.05 *M*. The product purified by this method on a 0.2-l scale represented a yield of about 40% α_1-PI based on 1.3 mg α_1-PI/ml of human plasma [4]. Chan et al. [10] demonstrated the use of gel filtration (1.8-l), diethylaminoethyl (DEAE) chromatography (0.2-l) and hydroxyapatite chromatography (0.2-l) to purify α_1-

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PI from human plasma; a single band was shown on a disc polyacrylamide gel. The recovery of α_1-PI was about 10%. Crawford [11] first precipitated plasma (0.3-l) with ammonium sulfate before purifying α_1-PI with DEAE, quaternary aminoethyl (QAE), and gel filtration chromatography. This process yielded α_1-PI with a specific activity of 0.56 with 25% recovery.

Since 1974, several affinity ligands have been explored [12–16]. Pannell et al. [12] used blue dextran as the first α_1-PI capture step from plasma (0.2 l), followed by ammonium sulfate fractionation, DEAE chromatography at pH 8.8, and DEAE chromatography at pH 6.5. The purity was nearly 95% by isoelectric focusing in acrylamide gels, and the yield was 60% after these four steps. Thiomatrix was used to bind α_1-PI by an SH–SS disulfide interchange reaction [13]. In another study, triazine dye was shown to be a better affinity ligand than blue dye [14]. The combination of triazine dye affinity chromatography, ion-exchange chromatography, and gel filtration chromatography was required to remove the impurities. A one-step purification from plasma was achieved using monoclonal antibodies to α_1-PI [15]. The specific activity was increased 61-fold with a yield of 20%.

Cohn et al. [17] developed a plasma fractionation scheme in 1946 based on differential precipitation in alcohol that has been widely used in the plasma industry. Several studies have been performed using one of the Cohn fractions as the starting material [18–22]. Glaser et al. [18] used Cohn fraction IV-1 paste (50 g) as starting material. After solubilization of α_1-PI, purification was achieved with successive additions of a fumed silica, dithiothreitol, ammonium sulfate and DEAE chromatography; the yield of this process was 45% and purity was around 70%. α_1-PI has also been purified from Cohn fraction IV-1 paste by polyethylene glycol (PEG) precipitation and DEAE chromatography [19–21]. One viral inactivation step, i.e., heating at 60°C for 10 h, has been incorporated into the latter process. Clearance of viruses has also been achieved by a PEG precipitation process step [23]. The latter process is the only method currently approved for α_1-PI production for use in human therapy [22]. Purification of α_1-PI from Cohn fraction II+III (equivalent to Cohn effluent II+III) was demonstrated by Burnouf et al. [24]; this process included DEAE chromatography (2×20-l stacks), gel filtration chromatography (4×20-l stacks), and one heat viral-inactivation step. The recovery of α_1-PI was 65–75% with 80–90% purity, as determined by nephelometry.

Recombinant human α_1-PI has been produced from *E. coli*, yeast and from transgenic sheep [25–27]. Travis et al. [28] reported a purification procedure for recombinant α_1-PI from yeast similar to that described by Potempa et al. [3] for α_1-PI from human plasma at 0.35-l scale. A combination of PEG precipitation, DEAE chromatography, thiol-exchange chromatography, heparin chromatography, Zn-chelate chromatography, and aminohexyl chromatography was used by Bollen et al. [29] to purify yeast-derived human α_1-PI at the bench scale. Kwon et al. [30] used ammonium sulfate fractionation, protamine sulfate treatment and anion-exchange chromatography to purify recombinant yeast-derived α_1-PI; a yield of 16% was obtained.

In summary, there are no currently available methods that have been demonstrated to produce highly purified and virally inactivated α_1-PI with high yield on a commercial scale. We developed a new small scale process starting with Cohn fraction IV-1 paste [31]. The IV-1 paste was resuspended and processed by DEAE chromatography, cation chromatography, tri-*n*-butyl phosphate (TNBP)–cholate treatment, a second cation chromatography, and dry-heat treatment. This process has a recovery of 64–70% and purity above 95% by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Two independent viral inactivation steps were included to assure safety. In this paper, we describe the scaling-up of this process to 50-l columns, the removal of lipid, material balance, and other key parameters for establishing a large-scale, commercially feasible method.

2. Materials and methods

2.1. Equipment and materials

The chromatography steps were performed using a custom-built skid by SRT (Morrisville, NC, USA). All 75 cm×45 cm diameter bio-process glass (BPG)

columns were purchased from Pharmacia (Piscataway, NJ, USA). The DC10 and DC30 ultrafiltration/diafiltration (UF/DF) systems were from Amicon. The production scale freeze–dryer was manufactured by Stokes (USA).

DEAE-Sepharose Fast Flow was obtained from Pharmacia. Macro-Prep High sulfopropyl (S) resin was obtained from Bio-Rad (Hercules, CA, USA). Spiral media filter was purchased from Amicon. Milliguard cartridge 0.2 μm filter (CWSS) and 0.1-μm filters were provided by Millipore (Bedford, MA, USA). Manufacturer's names and catalog numbers of the following chemicals were: Tris (Amresco, Solon, OH, USA, No. 0497), dibasic sodium phosphate (J.T. Baker, Phillipsburg, NJ, #3827), sodium chloride (Morton, Chicago, IL, USA, No. 1388), sodium hydroxide (J.T. Baker, No. 3728-R), acetic acid (Fisher Scientific, Pittsburgh, PA, USA, No. A490-212), sodium citrate (Haarman and Reimer, Springfield, NJ, USA), TNBP (Fisher Scientific, No. 41311-2C), cholate (ICN Biomedicals, Costa Mesa, CA, USA, No. 02-102897), sucrose (Pfanstiehl, Waukegan, IL, USA) and histidine (Ajinomoto, Burlingame, CA, USA).

2.2. Large-scale purification of α_1-PI

Fig. 1 outlines the steps in processing α_1-PI from Cohn fraction IV-1 paste. Fraction IV-1 paste is suspended in a 0.01 *M* Tris buffer at 5°C, and the pH is adjusted between 9.25 to 9.50. Then the material is heated to 40°C for 1 h and cooled to 5°C. The heat-treated IV-1 suspension is adjusted to a conductivity ≤5 mS/cm with NaCl, and then the pH is adjusted to 8.0. The column, described below, is pre-equilibrated with 1 column volume (CV) of 0.1 *M* Tris (pH 8.0) containing 1 *M* NaCl and then equilibrated with about 3 CVs of 20 m*M* Tris (pH 8.0) containing 41 m*M* NaCl. Four CVs of IV-1 suspension were applied to each CV of DEAE-Sepharose Fast Flow resin. The suspension was loaded at a linear flow-rate of 150 cm/h to a 30 cm long×45 cm diameter column, followed by a 2-CV wash with equilibration buffer. α_1-PI was eluted with 20 m*M* dibasic sodium phosphate (pH 8.0) containing 95 m*M* sodium chloride. The bound proteins were eluted from the resin with 2 CVs of 1.5 *M* sodium chloride at pH 8.0. The column was cleaned with 3 CVs of 0.5 *M* NaOH, and the resin was stored in 0.02 *M* NaOH.

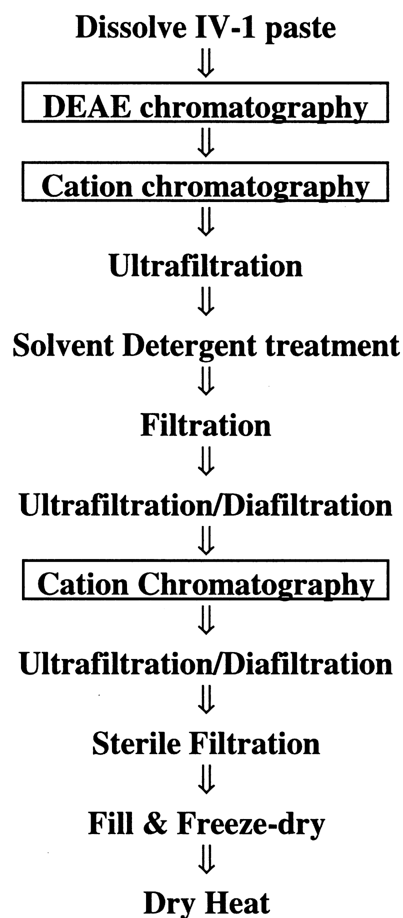

Fig. 1. Process flow for α_1-PI purification from Cohn fraction IV-1 paste.

The DEAE eluate was diluted directly to a conductivity of 3.0–3.5 mS/cm. The diluted eluent was adjusted to a pH range of 5.45 to 5.50. A 45 cm diameter column, packed 30 cm high with Macro-Prep High S cation resin, was pre-equilibrated with 0.3 CVs of 0.1 *M* sodium citrate at pH 4.5 and then equilibrated with 1 CV of 15 m*M* sodium citrate at pH 5.5 at about 20°C. Three CVs (equivalent to IV-1 suspension) of DEAE eluate was loaded at a linear flow-rate of 150 cm/h. α_1-PI passed through this column without binding. The flow-through, contain-

ing α_1-PI, was concentrated to an A_{280} of 30–40, adjusted to pH 7.0 with 1 *M* NaOH, and sodium chloride was added to a final concentration of 0.15 *M*. The bound proteins were eluted from the cation column with 2 CVs of 0.5 *M* NaOH. The column was stored in 0.02 *M* NaOH.

As a hold step, the α_1-PI was frozen at −30°C. For the TNBP–cholate treatment, α_1-PI was first thawed, and sucrose and citrate were added as stabilizers to a final concentration of 37% and 0.1 *M*, respectively. TNBP and sodium cholate were added from a stock solution of 5% (w/w) TNBP and 4% (w/w) cholate to a final concentration of 0.3% (w/w) TNBP and 0.2% (w/w) cholate. This solvent detergent treatment inactivates enveloped viruses [31]. After a 3-h incubation at 30°C, the TNBP and cholate were removed by filtration and then diafiltration.

The virally-inactivated cation 1 flow-through was filtered through a CWSS and a 0.1-μm filter. After filtration, the solution was diafiltered against 8 m*M* sodium citrate at pH 6.5. The diafiltered solution was diluted with 8 m*M* sodium citrate to an A_{280} of 2.5, and then was adjusted to pH 5.50 with 1 *M* acetic acid. This solution (total A_{280} of 150–175, as determined by $A_{280}\times$volume of cation 1 flow-through (*L*) was loaded onto a second High S cation column, 30 cm long×45 cm diameter (50-l), to remove any remaining contaminants, including residual TNBP, lipids and any α_1-PI, which was denatured by the viral inactivation step. The native, but not denatured, form of α_1-PI passed through the column in the loading buffer. The collected flow-through was adjusted to pH 7. The α_1-PI was concentrated by ultrafiltration to about 35 mg/ml using a DC10 ultrafilter. After ultrafiltration and diafiltration, 0.2 *M* NaCl, 80 m*M* histidine was added. The solution was filtered through a 0.2-μm filter and filled into 50-ml molded glass vials. The following freeze–drying cycle was employed during lyophilization: frozen at a shelf temperature of −25°C for 2 h at atmospheric pressure; decreased shelf temperature to less than −40°C for another 2 h; decreased pressure to 220 mTorr for the remainder of the process during which time the shelves were heated to +10°C for 30 h before being maintained at +35°C for a further 24 h (1 Torr=133.322 Pa). After stoppering, the final container dry-heat treatment was performed at 80°C for 72 h.

2.3. Analytical assays

2.3.1. α_1-PI activity assay

α_1-PI activity was determined by inhibition of porcine pancreatic elastase using chromogenic substrate [succinyl-(alanine)$_3$-*p*-nitroanillide] (Sigma, St. Louis, MO, USA) at 405 nm [7]. The assay was performed using a Denly Wellprep 2000 workstation. The samples were prepared by Hamilton Dilutor-Micro Lab 500. The microtiter plates were read with Molecular Devices Thermomax microtiter plate reader with a 405 nm filter and SoftMax for Windows software version 1.0. Potency was expressed as milligrams of α_1-PI per ml sample. A Human Reference Standard plasma (Helena Labs., Beaumont, TX, USA) was used.

2.3.2. Nephelometry assay

Immunoreactive α_1-PI and other proteins were quantified using a laser nephelometer immunoprecipitation assay (Behring Diagnostics, Behringwerke, Marburg, Germany) according to the manufacturer's recommendations.

2.3.3. Biuret protein assay

Biuret protein assay [22] was also performed using a Denley Wellprep 2000 workstation. The protein concentration was expressed as milligrams of protein per ml sample. A Reference Standard albumin (Bayer, Clayton, NC, USA) was used. The specific activity of α_1-PI was calculated on the basis of α_1-PI activity assay and Biruet protein assay.

2.3.4. SDS-PAGE

SDS–PAGE was performed using a mini-gel system (Novex, San Diego, CA, USA) with precast 10% polyacrylamide gels. All gels were fixed in 10% trichloroacetic acid (TCA) and stained with a colloidal blue stain (Integrated Separation Systems, Natick, MA, USA). Densitometry was performed using an Ultrascan XL Laser Densitometer (Pharmacia Diagnostics, Piscataway, NJ, USA).

2.3.5. TNBP and cholate assays

An assay for TNBP was developed by Zunic and McAleese [32]. Samples containing <1 μg/ml TNBP were loaded onto a C_{18} solid-phase extraction (SPE) column. The hydrophobic TNBP partitions into the stationary phase while the hydrophilic

protein is washed through the column with water. TNBP and tri-*n*-pentylphosphate (TNPP), which was included as an internal standard, were eluted from the column with hexane and concentrated by evaporation. The TNBP concentration was then determined by gas chromatography (GC) using a nonpolar column and flame ionization detection.

An assay for cholate was also developed by Zunic [33]. Cholate in the sample was converted to cholic acid by dilution with hydrochloric acid. This diluted solution was passed through a trifunctionalized C_{18} SPE column. Cholic acid was retained on the SPE column while the protein in the sample was washed through with hydrochloric acid. The cholic acid was eluted from the SPE column with acetone and collected in a volumetric flask. Acetone was evaporated from the solution with a nitrogen stream evaporator, and the remaining solution was reconstituted with sodium hydroxide. Cholic acid is deprotonated at this high pH, and the cholate ion was separated on an anion-exchange column. Detection was accomplished by measuring the current from the oxidation of cholate with a pulsed electrochemical detection system.

2.3.6. Cholesterol assay

An enzymatic spectrophotometric measurement was achieved using the Sigma Cholesterol-50 Reagent (Cat. No. 352-50), following the manufacturer's procedure 352. The assay was calibrated with a 200 mg/dl Cholesterol Standard (Sigma Cat. No. C7921). This standard was diluted with water to 50, 100, 150 and 200 μg/ml, which yielded linear concentration vs. absorbance (500 nm) plots with a typical 0.99 correlation coefficient. One-hundred μl of the samples were mixed with 1 ml cholesterol-50 reagent, and incubated for 7 min at 35°C. Selected samples yielded concentrations quite close to that determined by GC. A fresh reagent solution was used for the calibration and kept on ice during the assays.

3. Results

3.1. Optimization of DEAE chromatography at 10-l and 50-l scales

An optimum of 4 CVs loading of dissolved fraction IV-1 paste per CV of DEAE Sepharose was established previously by Chen et al. [31]; however, the optimal volumes of washing and elution were not determined. The pre-equilibration, equilibration, loading, washing, elution and cleaning buffers (see Section 2.2) were selected based on α_1-PI recovery, cost of chemical handling and water consumption.

Fig. 2 shows the effect of extending the wash and elution conditions on the recovery of α_1-PI (bars) and lipid distribution (●). No detectable α_1-PI was found in the flow-through or in the first CV wash (0–275 l). Transferrin eluted during the first 2 CV washes (180–305 l). Consequently, a 2-CV wash was selected, starting at 225 l and ending at 325 l. When a 2.5 CV elution was applied (450–575 l), about 95% of the active α_1-PI, determined by α_1-PI activity assay, was eluted with a buffer of 20 m*M* sodium phosphate and 95 m*M* sodium chloride. The volume of elution was determined by a target of 95% recovery. The high α_1-PI concentration, determined by nephelometry assay to be present in the cleaning buffer (Fig. 2, clean section), appears to be inactive α_1-PI because no activity was shown by the activity assay.

3.2. Optimization of the first cation chromatography

An optimum of pH 5.5 was previously determined for the first cation chromatography step [31]. An optimum conductivity for dilution was determined by testing 2.5, 3.0, 3.5, 4.0 and 4.5 mS/cm (data not shown). A range of 3.0 to 3.5 mS/cm was selected based on a target recovery of 90%. The low conductivity (2.5 mS/cm) appeared to have a low α_1-PI recovery (75–80%), but the high conductivity (4.0 and 4.5 mS/cm) resulted in a significant increase of immunoglobulin A (IgA) level.

Two buffers, sodium phosphate and sodium citrate, were considered. Five CVs of sodium phosphate buffer were needed for the equilibration step. In contrast, only 0.3 CVs of 0.1 *M* sodium citrate were necessary for pre-equilibration, and 0.8 CVs of 15 m*M* sodium citrate for equilibration were needed.

3.3. Optimization of the second cation chromatography

The conditions of the second cation chromatog-

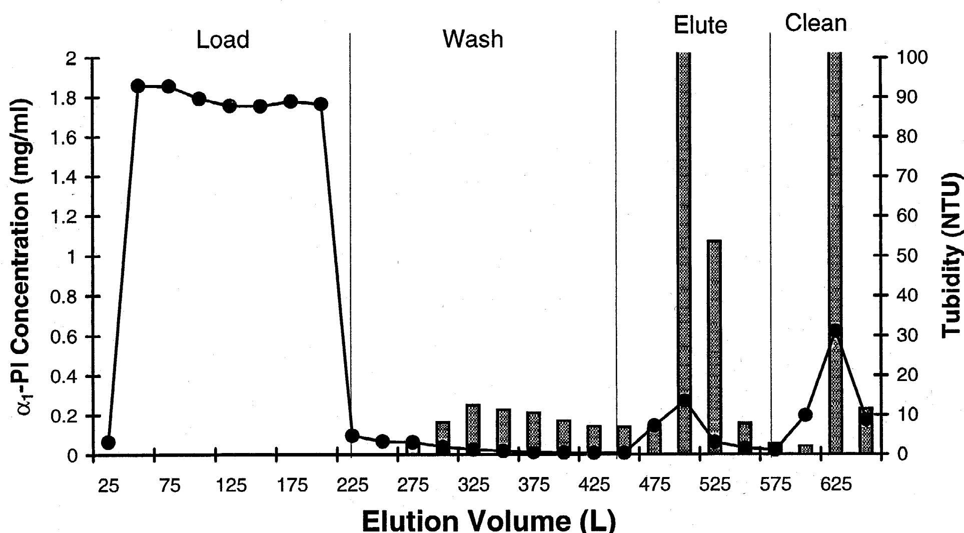

Fig. 2. DEAE chromatography of α_1-PI for the optimization of the process parameters at the 50-l scale. Samples were taken every half-column volume (CV) (equivalent to 25 l). The bars represent α_1-PI concentration measured by nephelometry assay, as shown on the left ordinate. The dots on the line indicate turbidity measurements, an indicator of lipid concentration, as shown on the right ordinate. Chromatography conditions were as described Section 2.2 with 4 CV loading, 5 CV wash, 3 CV elution and 2 CV cleaning. α_1-PI measured by nephelometry includes both active and inactive forms.

raphy were similar to those of the first cation chromatography step, except for the nature and amount of starting material. Only α_1-PI, residual IgA, and α_1-acid-glycoprotein were detectable by nephelometry after the second cation chromatography. α_1-Acid-glycoprotein was eluted at a constant concentration regardless of the amount of loading. α_1-PI specific activity dramatically decreased as the loading increased from 3 to 5 A_{280}/l solution/l resin (Fig. 3). The IgA concentration in the flow-through increased linearly with the amount of loading material. A loading of 3 A_{280}/l solution/l resin was selected to ensure a specific activity for α_1-PI of $\geq$0.9 and IgA of <1.8 mg/ml.

3.4. Cholesterol, TNBP and cholate removal

A low-lipid content was necessary for clarity and stability of the final product. Cholesterol concentration was used as a marker for lipid removal. Fig. 4 shows the cholesterol retention across the whole process from IV-1 paste. DEAE chromatography had a 49% cholesterol retention. This was further decreased after cation 1 chromatography to 17% and after cation 2 chromatography to less than 0.1%.

Filtration, with a CWSS filter followed by a 0.1-μm filter (see Section 2.2) after the TNBP–cholate treatment, showed an 89% cholesterol elimination, which proved to be a critical step for the cholesterol removal. The capacity of the CWSS filter was found to be 1.5 l/ft.^{2}, and the capacity of the 0.1-μm filter was 0.5 l/ft.^{2} (1 ft.=30.48 cm).

TNBP and cholate were mainly removed by diafiltration following the filtration with CWSS filters. The concentration of TNBP was reduced from 3480 μg/ml to 7.2 μg/ml and the cholate level from 3200 μg/ml to 4 μg/ml following diafiltration against 8 volumes of 8 m*M* sodium citrate at pH 6.5. The residual TNBP and cholate were removed by both cation 2 chromatography and the final 0.2 *M* NaCl diafiltration step. The final bulk concentration of TNBP and cholate were 0.5 μg/ml and <2 μg/ml, respectively.

3.5. 50-l scale process

A 50-l scale experiment was performed using the following conditions. The 280 nm absorbance profiles of the DEAE, cation 1, and cation 2 runs are shown in Fig. 5A–C. The flow-through during the

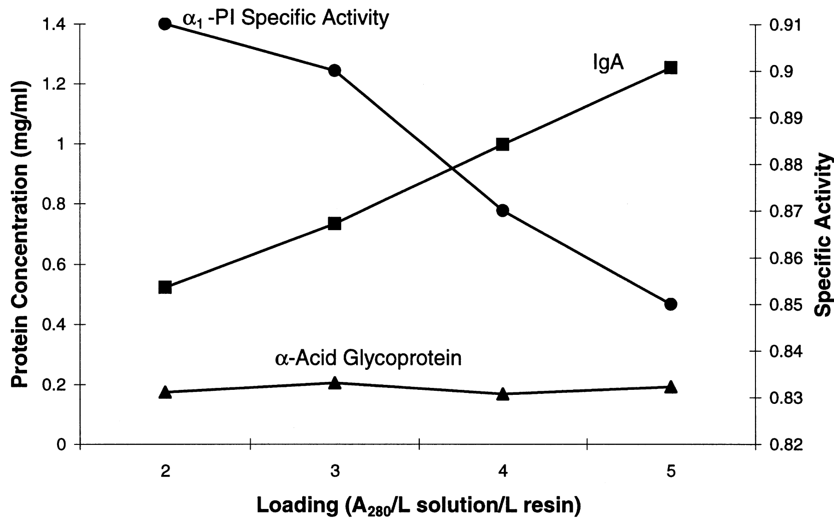

Fig. 3. Effect of different loading volumes on the purity of α_1-PI from the second cation column. Loading is varied by total amount of A_{280}/l solution/l resin. α_1-PI specific activity is represented by (●) with the scale on the right ordinate. The (■) represents IgA concentration, using the left ordinate and (▲) represents α_1-acid-glycoprotein concentration, using the left ordinate. Specific activity is calculated based on α_1-PI activity and Biuret protein assays. IgA and α_1-acid-glycoprotein concentrations are measured by nephelometry. α_1-PI, IgA and α_1-acid-glycoprotein are the only three proteins above the detectable limits of the nephelometry assay after the second cation chromatography.

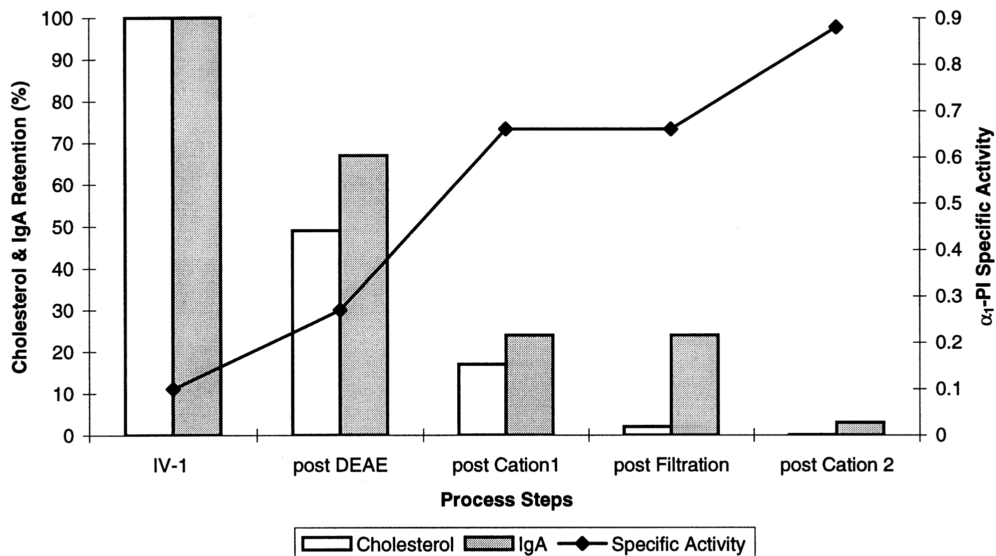

Fig. 4. Cholesterol and IgA retention, and α_1-PI specific activity across each process step. The white bars represent cholesterol retention. The black bars represent IgA retention. The line represents α_1-PI specific activity. The cholesterol concentration was determined as described in Section 2.3.6 and is used as an indicator of total lipid content. The specific activity was calculated based on α_1-PI activity and Biuret protein assays. IgA concentration was measured by nephelometry.

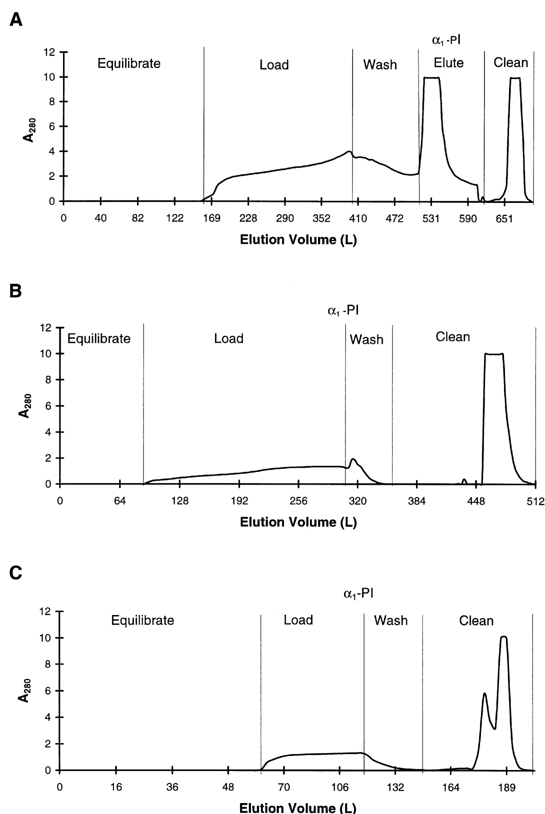

Fig. 5. The absorbance profiles for DEAE chromatography, cation 1 chromatography, and cation 2 chromatography at the 50-l scale. The absorbance profile (280 nm) for the DEAE column is given in (A). (B) Shows absorbance profile for purification of α_1-PI by the first cation column; and (C) shows absorbance profile for purification of α_1-PI by the second cation column. Equilibration, load, wash, elution and clean portions of each are divided by lines, and were performed as described in Section 2.2. "α_1-PI" indicates the elution of this protein following chromatography with DEAE (A) and its presence in the flow-through-wash using cation chromatography (B, C).

DEAE chromatography mainly consisted of lipoproteins (Fig. 5A). α_1-PI was eluted during the DEAE elution step (Fig. 5A). Native α_1-PI was collected as a combined flow-through-wash pool for the cation chromatography (Fig. 5B,C). Cleaning was performed under reversed-flow conditions for all three chromatography steps. The peaks eluted during the cleaning phase in Fig. 5B,C represented proteins bound more tightly to the resin, including denatured α_1-PI.

Table 1 shows the summary of the yield and the purity for this experiment. The DEAE chromatography step had a yield of 108% (±10%), which was consistent with the 10-l scale studies. A yield of 89% was achieved after the DEAE, cation 1, and ultrafiltration steps. The overall yield after UF/DF cation 2 was 76% with a purity of 0.87 g α_1-PI per g total protein. Filling and freeze–drying had a recovery of 93%. The total recovery from this process was 67%.

Fig. 6 shows a SDS–PAGE gel of the intermediates at different stages. The profile of the DEAE eluate looked similar to that of the IV-1 suspension (lanes 2 and 3). Lipids and lipoproteins are poorly detected by this analysis. The purity of α_1-PI after cation 1 was significantly increased (lanes 3 and 4). The SDS–PAGE scan indicated 98% purity after cation 2 before dry-heat treatment (lane 6). Some high-molecular-mass aggregates were formed during the dry-heat treatment (lane 7).

The purity of the final product was assessed by nephelometry, high-performance liquid chromatography (HPLC), non-reduced SDS–PAGE and specific activity (Table 2). Nephelometry and SDS–PAGE measure both the native and the denatured α_1-PI. However, the conformation of the native form is radically different from the denatured (inactive) form. This difference can be resolved by size exclusion HPLC. Thus, measurements of purity based on HPLC and specific activity measurements are generally lower than those obtained by nephelometry or SDS–PAGE techniques. Minimal amounts of immunoglobulin G (IgG), IgA and α_1-acid-glycoprotein were found by the nephelometry assay. The specific activity as deduced from the enzyme inhibition assay correlated with the amount of monomer present in the HPLC assay.

Table 3 lists each chemical requirement for producing 1 kg α_1-PI from this process. These requirements reflect the operating cost. They are based upon an overall yield of 67%.

4. Discussion

We have described a new process for producing highly purified (>95% by SDS–PAGE) α_1-PI on a large scale. This contrasts with earlier methods which were performed predominantly at small scale [9–15,25–30] or produced significantly less pure

Table 1
Recovery of α_1-PI from manufacturing scale (50-l column) process[a]

Step	Total Total α_1-PI (g)	Total protein (g)	Yield from IV-1 suspension (%)	Yield from previous step (%)	Specific activity (g α-PI/g protein)
Dissolved IV-1 paste	373	3408	100		0.11 (0.10–0.14)[b]
DEAE eluate	404	1600	108	108	0.28 (0.26–0.29)
Cation 1 and UF cation 1	332	549	89	82	0.68 (0.52–0.78)
S/D and filtration and UF/SF S/D	314	438	84	95	0.61 (0.61–0.72)
Cation 2 and UF/DF cation 2	284	326	76	90	0.87 (0.86–0.90)
Filling and freeze–drying	265	312	71	93	0.85 (0.85–0.89)
Dry-heat	250	302	67	94	0.83 (0.83–0.89)

[a] The results are given from one representative run at the manufacturing scale. Recoveries of individual steps are consistent with experiments performed at the pilot scale.
[b] Parentheses indicate the range obtained from 10-l scale studies.

protein [17–22,24]. Specifically, this process shows good recovery (67%) from IV-1 paste, includes 2 viral inactivation steps and is relatively easy to automate.

Cohn fraction IV-1 paste was chosen as the starting material despite the 51% loss of activity associated with this precipitation step and subsequent solubilization (Table 4). This choice was made largely because processing of this paste does not affect licenses to other existing products. The dissolved paste can be applied directly onto the DEAE column (Figs. 1 and 2, Table 2). The function of the DEAE resin is primarily to capture α_1-PI and to remove lipoproteins and a significant amount (60%) of other impurities in the flow-through, wash and cleaning steps (Fig. 2). Elution is achieved with 20 m*M* sodium phosphate and 95 m*M* NaCl at pH 8.0. Buffer conditions were optimized with respect to the

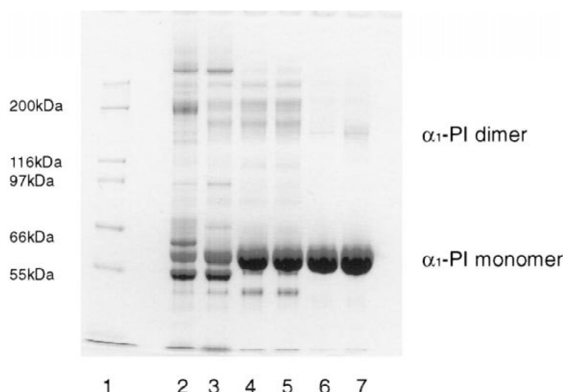

Fig. 6. Non-reduced SDS–PAGE analysis of intermediates from the 50-l column experiment. Samples were as follows: molecular mass markers (lane 1), IV-1 suspension (lane 2), DEAE eluate (lane 3), cation 1 flow-through-wash (lane 4), TNBP–cholate-treated cation 1 flow-through-wash (lane 5), cation 2 flow-through-wash (lane 6), and dry-heat-treated cation 2 flow-through-wash (lane 7). Protein (20 μg) were loaded in each lane and visualized by Coomassie blue stain.

Table 2
Purity of dry-heat treated cation 2 flow-through-wash

Test	α_1-PI monomer (%)	Concentration (mg/ml)
Nephelometry		
α_1-PI		25.2
IgG		0.004
IgA		1.07
α_1-Acid glycoprotein		0.13
Transferrin		<0.09
Albumin		<0.02
IgM		<0.05
Haptoglobin		<0.06
Ceruloplasmin		<0.02
A1 apolipoprotein		<0.05
Fibrinogen		<0.13
Fibronectin		<0.02
AT-III		<0.03
HPLC		
α_1-PI monomer	87	
Non-reduced SDS–PAGE		
α_1-PI monomer	95	
Specific activity		
Active α_1-PI	83	

The results are from one representative experiment performed at the manufacturing scale.

Table 3
Materials required for 1 kg α_1-PI product

Chemical names	Amount of each chemical (kg)
Tris	8.95
Sodium chloride	89.42
Sodium phosphate dibasic	2.62
Sodium hydroxide	46.8
Acetic acid	0.5
Sodium citrate	15.26
Phosphoric acid	3.0
Sucrose	26.4
TNBP	0.3
Sodium cholate	0.2
Histidine	0.34
Water	22 400

The total amount of each chemical is calculated based on 65% recovery from dissolved VI-1 paste to the end of dry-heat treatment.

yield of α_1-PI. The performance of the resin, such as A_{280} trace, nephelometry profile of DEAE eluate, α_1-PI purity, and yield, was predictable upon scale up from 10 l [31] to 50 l (Fig. 5). The DEAE Fast Flow resin could be cleaned and sanitized by 0.5 *M* NaOH and regenerated by 0.1 *M* Tris and 1 *M* NaCl. Water consumption was minimized by using this regeneration step before equilibration.

Following anion-exchange chromatography, the eluate containing α_1-PI was diluted before being applied to the cation column. This could be achieved either by diluting it in line with water in a ratio of about 1:3 or it could be diafiltered against six volumes of 15 m*M* sodium citrate to achieve the correct ionic strength of 3.0–3.5 mS/cm. By either method the amount of material applied to the column was found to be optimal at about 12 g protein/l resin. Major impurities at this stage were IgA, α_1-acid-glycoprotein, antithrombin III (AT-III), A-1 lipoprotein and albumin. For cation-exchange chromatography, four different resins were tested. Poros 50 HS appeared to have a lower capacity than Macro Prep HS, while SP Sepharose HP and SP Sepharose Fast Flow had a lower purity of α_1-PI as determined by SDS–PAGE. The column bed height and linear flow velocity were retained during scale-up.

The next step of the process involved viral inactivation using TNBP and cholate. The concentrations of these chemicals (0.3% and 0.2%, w/w, respectively) were chosen to assure complete inactivation of enveloped viruses as predicted by the model viruses, bovine viral diarrhea virus and pseudorabies virus. Sugars such as sucrose had previously been found to stabilize α_1-PI [19] during heat treatment, and were also found to act as α_1-PI stabilizers during solvent detergent treatment [31]. The removal of these chemicals was necessary and was achieved by diafiltration and filtration.

Table 4
α_1-PI recovery from both Cohn fractionation and this process

Steps	Recovery from plasma[a] (%)	Total α_1-PI (kg)
Plasma	100	3.1 (2400 l plasma)
Effluent I	95	2.9
Effluent II+III	87	2.7
IV-1 Paste	18	0.56
Dissolved IV-1 paste	49	1.5
Dry-heat treated α_1-PI	32	1

[a] The results for plasma, effluent I and effluent II+III are provided by Wytold Lebing (unpublished observation). The results for IV-1 dissolution and dry-heat treated α_1-PI are calculated based on 1.3 mg α_1-PI/l plasma.

The diafiltered material was then passed through a second cation column. The conditions were optimized to produce a specific activity ≥0.9 and IgA concentration <1.8 mg/ml for the final product (Fig. 3). Residual α_1-acid-glycoprotein is not removed at this step due to its low isoelectric point (pI=2.7) and negative charge at pH 5.5 [34]. As a result, it coelutes with α_1-PI in the flow-through and wash. The second cation column also serves as a removal step for the denatured α_1-PI which binds to the resin [31], residual TNBP and cholate and lipoproteins.

The final steps of processing involved formulation, bulking, lyophilization and terminal heat treatment. Optimization of these conditions are not described in the text though the use of histidine as a thermal stabilizer is described by Chen et al. [31]. The final α_1-PI product achieves a purity of 95% monomer by non-reduced SDS–PAGE with a specific activity of 84%. The clarity by T_{580} is 98% with 0.5% aggregates measured by HPLC. In addition, all the chemicals (Table 3) used in this process have passed the material clearance studies.

In all, the purification process presented above has significantly improved both the yield and the purity of α_1-PI as compared to the previous commercial process [22]. The processing time of DEAE chromatography is 4.5 h, first cation, 2.6 h, and second cation, 1.8 h. These times permit effective cycling of the columns for large-scale manufacturing. The overall process time can only be determined upon the completion of the equipment design.

The availability of IV-1 paste from human plasma still limits the availability of α_1-PI product to patients. Several alternative sources of human α_1-PI have been pursued. Recombinant human α_1-PI has been expressed in *E. coli* and yeast [28,30]. The thermal deactivation half-lives for the *E. coli* α_1-PI, the yeast α_1-PI and plasma α_1-PI are 1.42, 10.7 and 26.2 min, respectively, at 58°C [30], indicating that structural differences may exist. In addition, known differences in glycosylation may affect the bioavailability of the recombinant proteins. Another approach to increase the production of α_1-PI has been to express it in the milk of transgenic sheep [27]. Significant progress has been made in the area of human protein expression in animals, although possible differences in glycosylation patterns still exist. This technology also incurs extensive purification costs due to the need for very high purity to resolve possible issues of antigenicity of residual animal proteins. The grave situation in the UK regarding probable transmission of transmissible spongiform encephalopathief (TSE) by bovine spongiform encephalopathief (BSE)-contaminated, bovine-derived products highlights the need for the development of prion elimination methods for transgenic products.

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